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(54) **PROCEDE MODIFIE DE PRODUCTION D'ACIDES NUCLEIQUES MODIFIES AU PHOSPHORAMIDITE**

(54) **MODIFIED PHOSPHORAMIDITE PROCESS FOR THE PRODUCTION OR MODIFIED NUCLEIC ACIDS**

(57) A modified phosphoramidite process for the synthesis of nucleotide sequences. By use of a modified nucleoside phosphoramidite it is possible to produce nucleotide sequences which have a modified phosphate residue.

ABSTRACT

A modified phosphoramidite process for the synthesis of nucleotide sequences. By use of a modified nucleoside phosphoramidite it is possible to produce nucleotide sequences which have a modified phosphate residue.

The invention relates to a modified phoshoramidite process for the production of modified nucleic acids and new compounds which are used in this process.

Nucleic acids are a group of compounds which are of fundamental importance for life in the world and are therefore present in all organisms. The genetic information is stored in them. They are also a criterium for the differentiation and identification of different species of organisms, since the nucleic acid sequences are characteristic for each organism. There has therefore been no lack of attempts to synthesize as well as to detect nucleic acids.

Nucleic acids can be synthesized chemically or enzymatically. The chemical synthesis of the naturally occurring nucleic acids in the β configuration has recently gained even more in significance since such large amounts of nucleic acids with a defined nucleotide sequence can be produced. The chemical synthesis has been able to establish itself in particular for the synthesis of oligonucleotides in the β configuration. The different methods can be distinguished according to

the type of the nucleotide building blocks used and the reaction steps for attachment to the neighbouring nucleotide in the sequence:

In the phosphodiester method a nucleoside monophosphate, in which all reactive groups with the exception of the phosphate residues are protected, together with a coupling agent, for example a trialkylarylsulphonic acid chloride, is reacted with a further nucleoside in which all reactive groups, except for the hydroxy group at which the reaction is to take place, are protected. The yields in this method are low, primarily because during the condensation steps to build the oligonucleotide chain undesired side reactions occur at the non-esterified OH-groups of the internucleotide (phosphate) linkages and result in complex reaction mixtures. Furthermore, it has the major disadvantage that the phosphoric acid diesters formed are only soluble in a few protic solvents in which the esterification has to be carried out. Such solvents like pyridine, dimethylformamide or dimethylsulphoxide have well-known disadvantages such as e.g. high boiling points. As a result of the polar character of the phosphodiester derivatives the isolation and purification has to be carried out over ion-exchangers and cannot be carried out in a simple way e.g. over silica gel using solvents with low boiling points (such as e.g. dichloromethane).

The disadvantage of the insolubility of the products in a multitude of organic solvents is avoided by the phosphotriester method.

The phosphotriester method uses one phosphoric acid derivative which has only 1 reactive group, but 2 hydroxy groups next to the phosphorus atom which are

protected with different protecting groups. After the reaction with the first nucleoside one of the protecting groups is cleaved off and the hydroxy group formed can then be activated for the reaction with the second nucleoside. As a consequence of this procedure it is necessary to carry out two additional reaction steps on the nucleoside phosphate which leads to a reduction in the yield of activated nucleoside phosphate.

A particularly advantageous method which manages with fewer reaction steps on the relatively expensive synthetic building blocks has become known as the phosphoramidite process (Gait, M.J. et al., Oligonucleotide Synthesis: A Practical Approach, IRL Press Oxford). In this procedure no phosphoric acid derivatives but rather derivatives of phosphorous acid, the so-called phosphoramidites, are used. The following residues are attached to the trivalent phosphorus atom:

- a reactive group, for example a halogen atom, which enables the linkage with the first nucleoside,

- a secondary amino group with which the linkage with the second nucleoside can be effected after activation and

- a hydroxy group masked by a protecting group.

In the first step of the phosphoramidite process the phosphorous acid derivative is reacted with a first nucleoside; in this process the nucleoside replaces the reactive group. In the second step the secondary amino group is replaced selectively by the second nucleoside. A tetrazole is usually used as the activating reagent in

the second step. In a subsequent step the nucleotide sequence is oxidized, for example with iodine, and the protecting group is cleaved off. In one variant, the phosphoramidite process has been described as a solid phase process. In this variant the growing nucleotide sequence is bound to a solid phase. The separation of excess synthetic reagents and building blocks as well as the purification of the oligonucleotide sequence is greatly simplified by this means. Commercially available automated nucleic acid synthesizers work according to this procedure. Their construction has e.g. been matched to specific steps of the phosphoramidite process.

Nucleic acids with a known nucleotide sequence have in particular been applied for the specific detection of DNA in biological sample material.

In such methods of detection use is made of the property that the single strands of nucleic acids can react with other single-stranded nucleic acids to form a double strand if the single strands have nucleotide sequences which are complementary to each other and both have the same configuration at the C-1 of the ribose (α or β). Since the nucleic acids which occur naturally have the β configuration with regard to the linkage of bases and sugars, the β nucleic acids in particular may be considered as complementary nucleic acids. The process of double strand formation is called hybridization.

The formation of a double strand can be detected if a modified single-stranded complementary nucleic acid is used for the hybridization with the single-stranded nucleic acid. Afterwards the amount of the hybridized nucleic acids is determined via the modification which can for example be a radioactive label.

For the synthesis of modified nucleic acids either a natural nucleic acid which is already available can be chemically or enzymatically modified or the nucleotide sequence can be synthesized with the aid of nucleotide building blocks which have already been modified.

However, by modifying the ends of already completely synthesized nucleic acids, as suggested for example for the 5'-end in WO 86/07363, nucleic acids can be prepared which only contain a single modified nucleotide per single strand. Methods for determining the amount of nucleic acids with this type of modified nucleic acids as probes are therefore less sensitive.

Therefore it was suggested in the EP-A 0173251 that the bases of complete nucleic acids be modified by chemical reactions. However, several reaction steps on the nucleic acid are necessary for this and the rate of modification is dependent on whether the nucleic acid contains bases with free amino groups, the modification of which does not impair the ability to hybridize with complementary nucleic acids.

The preparation of a dinucleotide which has a modification at the phosphorus atom is described in Jäger et al. (Biochemistry Vol. 27, p. 7237 (1988)). The modification consists of a primary amino group bound via a linker and is introduced into a process similar to the usual phosphoramidite process.

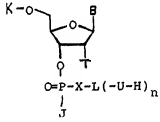
However, this process cannot be carried out on the usual automated synthesizers which use the phosphoramidite method. A further disadvantage is that no more additional nucleotides can be joined on because the free

amino group reacts with the electrophilic reagents which are used for this.

Each of the available state-of-the-art methods therefore has considerable disadvantages.

The present invention seeks to avoid the disadvantages of the known processes and in particular to make available a process for the synthesis on solid phases of nucleic acids in the β configuration which are modified at the phosphate residue which can be carried out with simple starting materials in few reaction steps with high yields.

In accordance with one aspect of the invention there is provided a process for the production of nucleotide sequences of the formula IX



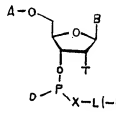
IX

in which

- K represents hydrogen or the phosphorus atom of the phosphate residue of a further nucleotide or of a nucleotide sequence,
- J represents a hydroxy group or a 5' oxygen atom of a further nucleotide or of a nucleotide sequence,
- B represents a natural or modified nucleobase,
- T represents hydrogen, lower alkyl, azide, lower alkyloxy or a hydroxy group,

X represents oxygen or sulphur,
L represents a (n + 1) valent bridging link,
U represents oxygen, sulphur, nitrogen or N-H and
n represents a natural number from 1 to 200,

by reaction of a nucleoside phosphoramidite with a further nucleotide which has a free hydroxyl group and oxidation of the nucleotide sequence formed to a phosphate wherein a compound of the formula I



(I)

is used as the nucleoside phosphoramidite in which

A represents an oxygen protecting group, a nucleotide or an oligonucleotide,
B represents a natural or modified nucleobase,
X represents oxygen or sulphur,
L represents a (n + 1) valent bridging link,
T represents hydrogen, lower alkyl, N_3, lower alkoxy or a hydroxy group which is protected if desired,
U represents oxygen, sulphur, nitrogen or N-H,
V represents a protecting group which can be cleaved off,
n represents a natural number from 1 to 200 and
D represents a secondary amine residue.

The lower alkyl and lower alkoxy groups contain 1 to 6, preferably 1 to 4 carbon atoms.

Processes for the production of nucleic acids by the so-called phosphoramidite process are known in principle, for example from Biochimie 1985, 67, 673-684. The process of the present invention differs in particular from the state-of-the-art processes in that another nucleoside phosphoramidite, namely that of formula I, is used as the starting material.

A preferred residue A in formula I is an oxygen protecting group. Protecting groups which are suitable for the protection of the 5'-hydroxy group in nucleotide syntheses are known. Protecting groups such as the triphenylmethyl group or the dimethoxytriphenylmethyl group which can be cleaved off under acidic conditions are used particularly often.

If the residue A represents a nucleotide or oligonucleotide it can either be a natural or a modified nucleotide or oligonucleotide. The nucleotides are preferred to the oligonucleotides because the synthesis using oligonucleotides is more laborious. The nucleotides or oligonucleotides of the residue A can also be residues prepared according to the invention. Reactive groups of the nucleotides or oligonucleotides of the residue A are preferably protected by suitable protecting groups. In particular the terminal 5'-hydroxy group of the nucleotide or oligonucleotide of the residue A is protected by an oxygen protecting group. This oxygen protecting group has in particular the meaning mentioned above for residue A.

The natural nucleobase of the residue B is preferably adenine, thymine, cytosine, uracil or guanine. The modified bases can for example be bases altered in their structure in the ring or in the substituents. Examples

are 7-deazaguanine or 5-aminoalkyluracil or 8-aminohexyl-amino-adenine. Those bases are preferred in which the Watson-Crick base-pairing with a complementary nucleic acid is not influenced or only to a very slight extent.

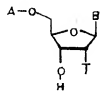
The residue T can have the ribo or arabino configuration. The ribo configuration is preferred. Groups which can be cleaved off under basic, acidic or nucleophilic conditions, preferably the t-butyldimethylsilyl or triisopropylsilyl group, are possible as the protecting group for the hydroxy residue.

The further nucleotide reacted with the nucleoside phosphoramidite of formula (I) is preferably bound to a solid phase or carrier.

The protecting group V is preferably a protecting group which can be selectively cleaved off. A protecting group is preferred which can be cleaved off simultaneously under the conditions under which the complete nucleotide sequence is cleaved from the solid carrier. Therefore protecting groups which can be cleaved off under acidic conditions, as denoted for instance in A, are not preferred. Particularly preferred are therefore protecting groups which can be cleaved off under alkaline or ammoniacal conditions; the fluorenylmethoxycarbonyl group or the trifluoroacetyl group have proven to be particularly favourable.

For the case in which A in formula (I) is an oxygen protecting group, this protecting group may be split off after the reaction of the compound of formula (I) with the further nucleoside; the resulting compound may then be reacted with a further mononucleoside phosphoramidite or a compound of the formula (I).

The compounds of the formula I can be produced from compounds of the formula II

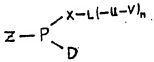


(II)

in which

A represents an oxygen protecting group, a nucleotide or an oligonucleotide,
B represents a natural or modified nucleobase and
T represents hydrogen, a (if desired, protected) hydroxy group, lower alkyl, N_3 or lower alkyloxy,

by reaction with phosphanes of the formula III



(III)

in which

Z represents a good leaving group,
X represents oxygen or sulphur,
L represents an at least bivalent bridging link,
U represents oxygen, sulphur, nitrogen or N-H,

V represents a protecting group which can be cleaved off,

n represents a natural number from 1 to 200 and

D represents a secondary amine residue.

The reaction conditions can be chosen by the expert analogous to those already described for the nucleoside phosphoramidites of the state of the art. However, in this process it must be taken care that no reagents are used which when used can cleave off the protecting group V. These reaction conditions are known to the expert for the individual protecting groups.

The phosphane of the formula III can be synthesized in a simple way from commercially available starting materials. In the preferred order of the production reactions the reaction with a secondary amine is scheduled first since this is a cheaper raw material. Thus in this reaction step losses in yield by unspecific reaction can if necessary be accepted. The phosphane of the formula III is preferably produced in that a compound of the formula (VI)

$$P(-Z)_3 \qquad \text{(VI)}$$

in which Z represents a good leaving group is reacted with a secondary amine of the formula (VII)

$$H\text{-}D \qquad \text{(VII)}$$

in which

D represents a secondary amine residue

and the product is allowed to react with the compound of the formula VIII

$$H\text{-}X\text{-}L(\text{-}U\text{-}V)_n \qquad (VIII)$$

in which

X represents oxygen or sulphur,
L represents a (n + 1) valent bridging link,
U represents oxygen, sulphur, nitrogen or N-H,
V represents a protecting group which can be cleaved off,
n represents a natural number from 1 to 200

and the product formed is isolated.
The residue Z is preferably halogen and particularly preferably chlorine.

Compounds of the fomula VII are in particular secondary amines known to the expert of the formula $H\text{-}NR^1R^2$ in which R^1 and R^2 are the same or different and primary, secondary or tertiary alkyl residues with 1-10 carbon atoms or together, if desired, represent an alkyl-branched cycloalkyl residue, the cycloalkyl residue having 5-7 carbon atoms and optionally containing one or two nitrogen, oxygen and/or sulphur atoms as hetero-atoms; or $N\ R^1\ R^2$ represents an imidazolyl, triazolyl, tetrazolyl, 3-nitro-1,2,4-triazolyl, thiazolyl, pyrrolyl, benztriazolyl or benzhydroxytriazolyl residue. Diisopropylamine and morpholine have proven to be particularly preferable amine.

Linear or branched, saturated or unsaturated hydrocarbons with 1-10, preferably 2 to 6 carbon atoms are worthy of mention as the bridging link. The hydrocarbon chain can be interrupted by heteroatoms, for example oxygen or sulphur. The bridging link can also contain aliphatic or aromatic ring systems. The bridging link can also have further heteroatoms. However, with regard to the reactions which are to be carried out in the process according to the present invention with compounds which contain this bridging link, those bridging links must, however, be excluded which have free unsubstituted or primary amino groups or hydroxy groups as substituents. The bridging link is connected through n covalent bonds with n groups U. The integer n has a value of 1 to 200, preferably 1 to 3, more preferably 1.

The compounds of the formula III have the advantage compared to the phosphanes of the state of the art that they can be used for the synthesis of nucleoside phosphoramidites for the phosphoramidite synthesis of nucleic acids as well as having a reactive group in a protected form; this can serve as a linkage site for detectable residues.

The process according to the present invention for the production of nucleotide sequences includes in particular the following steps:

- Coupling reaction of a nucleoside phosphoramidite of the formula I with a nucleoside which has a free hydroxy group. The nucleoside with the free hydroxy group is preferably bound covalently to a solid carrier. The other reactive groups of the nucleoside such as amino groups, carbonyl groups or further hydroxy groups are preferably protected by

protecting groups which are stable under the conditions of the coupling reaction. Preferably a 2'-hydroxy group which may be present on the sugar residue is protected by a t-butyldimethylsilyl group. The free hydroxy group is preferably the 5'-hydroxy group of the sugar residue.

The nucleoside can be a mononucleoside, an oligo- or polynucleotide. It is, however, preferably a mononucleoside, oligo- or polynucleotide of 2 to 200, preferably 20 to 60 nucleotide building blocks. The nucleotide building blocks can be natural or modified nucleotides.

The nucleoside can also be a nucleoside modified in a manner according to the present invention.

- Afterwards the nucleotide sequence bound to the solid phase is oxidized. Iodine has proven to be a preferred oxidizing agent.

- Subsequently a capping step is preferably carried out. This is carried out according to known methods.

- Selective cleavage of the protecting group A or the oxygen protecting group of the terminal 5'-hydroxy group of the nucleotide or oligonucleotide of the residue A. In the preferred case, if the oxygen protecting group of the residue A is a protecting group such as a dimethoxytriphenylmethyl group which can be cleaved off under acid conditions it can be cleaved off for example by dichloroacetic acid.

- These first steps can now be repeated, if desired. For this a conventional mononucleoside phosphoramidite or one having formula I can be used as the mononucleoside phosphoramidite.

- As soon as the desired length of the nucleotide sequence has been reached, the protecting groups V are cleaved off. In the case of the amino protecting group the trifluoroacetyl or the fluorenylmethoxycarbonyl residue (Fmoc) has proven to be particularly advantageous.

- Subsequently the nucleotide sequence is cleaved off from the solid carrier in a known way. The conditions are chosen according to the type of covalent bond and are not influenced by the modification according to the present invention.

  Those conditions are, however, particularly preferred under which the cleavage of the protecting group V and the cleavage of the nucleotide sequence from the carrier occur simultaneously. This can, for example, be effected by use of a carrier bound via a 3'-O-succinyl to CPG (controlled pore glass) and the Fmoc protecting group as residue V in which alkali, preferably concentrated aqueous ammonia solution or amine solution, is used as the cleavage reagent.

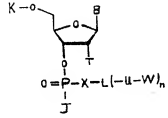
- Usually a purification step follows, for example a purification by means of HPLC chromatography or/and a dialysis. The same conditions apply here as those usually used for oligonucleotide synthesis.

All these steps have in common that, apart from the fact that another nucleoside phosphoramidite is used and that instead of the reagents to cleave off the oxygen protecting groups at the phosphate residue the prior art reagents for the cleavage of the protecting group V are used, no changes in the conventional course of the process need to be carried out. In particular the number of steps is the same as, or smaller than, in the conventional phosphoramidite process. Thus the process according to the present invention can be carried out in the available nucleic acid synthesizers for the phosphoramidite synthesis without changes in apparatus.

The nucleotide sequence of formula IX prepared in this way preferably has 2 to 200, particularly preferably 20 to 60 nucleotide building blocks. Of these 10 to 80 %, particularly preferably 20 to 50 %, of the nucleotide building blocks are nucleotide building blocks formed from the nucleoside monophosphates of formula I modified at the phosphorus atom. These modified nucleotide building blocks are preferably at an interval of 2-5 nucleotides to one another in the sequence.
The compounds of formula IX have many uses.

For example nucleotide sequences can be prepared in a simple manner from the nucleotide sequences of formula IX prepared according to the present invention which have a detectable residue or a residue which can be converted into a detectable residue. If the nucleotide sequence has several modified nucleotide building blocks, nucleotide sequences can be prepared which contain several such residues. This case is preferred since it has been proven that as a result the determination of nucleic acids becomes more sensitive.

In another aspect of the invention there is provided a process for the production of a nucleotide sequence of the formula V



(V)

in which

- K represents hydrogen or the phosphorus atom of the phosphate residue of a further nucleotide or of a nucleotide sequence
- J represents a hydroxy group or a 5' oxygen atom of a further nucleotide or of a nucleotide sequence
- B represents a natural or modified nucleobase
- T represents hydrogen, lower alkyl, azide, lower alkyloxy or a hydroxyl group
- W represents a detectable residue or a residue which can be converted into a detectable residue and X, L, U and n have the above-mentioned meaning,

wherein subsequent to the above mentioned steps the nucleotide sequence of formula IX formed is reacted with a compound of formula IV

Y-W (IV),

in which

Y is a reactive group and

W is a detectable residue or a residue which can be converted into a detectable residue.

In another aspect of the invention there is provided a nucleotide sequence of formula (V), as defined above.

A nucleophilic group which can be easily substituted or an electrophilic group are for example possible as the reactive group Y. Compounds of formula IV are for example carboxylic acid halides.

Electrophilic groups are for example the groups in activated esters or anhydrides. A preferred ester is for example the N-hydroxysuccinimide ester of haptens, if these have a carboxyl group.

The further nucleotide encompassed in the meaning of the residues K or J can be a natural or a modified nucleotide. The nucleotide sequence encompassed in the meaning of residues K or J can contain natural as well as modified nucleotide building blocks. The nucleotide sequence of formula V has preferably 2 to 200, particularly preferably 20 to 60 nucleotide building blocks. Of these 10 to 80 %, particularly preferably 20 to 50 %, of the nucleotide building blocks are nucleotide building blocks formed from the nucleoside monophosphates of formula I.

The residue W can be a low as well as a high molecular structure. Preferred low molecular reporter molecules are dyes and haptens; preferred high molecular groups are e.g. enzymes or immunologically active substances such as antigens or antibodies. Haptens are particularly preferred. Of these, those are particularly preferred which do not occur under normal conditions in body

fluids such as digoxigenin for example. Haptens and in particular digoxigenin have proven to be particularly advantageous as the immunologically active substance since the molecular weight of nucleotide sequences which have these is not changed much by the modification and can thus be used as a standard of length, for example, in gel chromatography.

It has turned out that the process according to the present invention for the production of nucleotide sequences has in addition the following advantages compared to the state of the art:

- Because the modification is introduced at the phosphorus atom the base-pairing of the nucleotide sequence formed with a complementary nucleotide sequence is not impaired.

- The nucleotide sequences formed are accepted as primers by polymerases.

- The modification can be introduced in addition to other modifications, for example, of the sugar residue or of the base, or in addition to 3'- or 5'-end labels.

- The process includes a convergent synthesis of the necessary building blocks. Such processes are particularly advantageous because the yields, in particular of the expensive nucleotide building blocks, can be kept high.

- The readily-available, naturally-occurring β nucleosides can be used for the synthesis of the nucleoside phosphoramidites.

- It was possible with the same or even a reduced number of reaction steps to utilize the well-known advantages of the solid-phase phosphoramidite process for the synthesis of nucleotide sequences in order to synthesize nucleotide sequences modified at the phosphate residue.

- Using the process according to the present invention it is possible to introduce a very special number of modifications at quite specific sites in the sequence.

- The modified nucleotide sequence formed can be used universally. For example different detectable residues can be chosen.

- Because the detectable residues are not present from the beginning in the nucleoside phosphoramidites, complications are avoided during the chemical synthesis of the nucleotides which would be expected when using enzyme labels or other sensitive reporter groups.

- The steric hindrance by reporter molecules can reduce the yield and efficiency of oligonucleotide syntheses. This disadvantage is avoided in the process according to the present invention.

The nucleotide sequences of formula V can be used advantageously as the nucleotide sequence complementary to the sample DNA in methods for the detection of nucleic acids in a sample by bringing the sample into contact with a nucleic acid which is essentially complementary to it, treatment of the mixture under conditions which lead to the hybridization of nucleic acids which are complementary to one another and detection of the detectable residue. The detection of the detectable residue can be effected by known methods. If the detectable residue is an immunologically active substance then the residue can be reacted with a labelled immunological partner. Afterwards the label is measured. In the case of this use of the nucleic acid according to the present invention haptens, in particular digoxigenin, are preferred as the residue W.

They are equally suitable as primers in the enzymatic synthesis of double-stranded nucleic acids from single-stranded nucleic acids. The double-stranded nucleic acid which forms then contains the nucleotide sequence in at least one of the two strands.

The invention is elucidated by the following Examples.

Example 1:

2-(9-fluorenylmethoxycarbonyl-)aminoethanol

68.0 g (ca 200 mMol) 9-fluorenylmethoxycarbonyl-N-hydroxysuccinimide ester (Fmoc-O-Su) is dissolved with stirring in 300 ml dioxan in a 1 l round-bottomed flask. 40 g Na_2CO_3 dissolved in 200 ml water as well as 14.4 ml (238 mMol) ethanolamine are added successively to the

clear solution. The pulpy reaction mixture which forms at once is stirred overnight at room temperature and is aspirated on the following day. The filtration residue which contains unreacted Fmoc-O-Su, N-hydroxy-succinimide as well as the desired product, is re-crystallized from acetic ester. After drying in a vacuum 47.4 g pure product = 76 % of the theoretical yield are obtained.

^{1}H-NMR (ppm) (DMSO):3.4 (m, CH_2O, 2 H); 3.6 (t, CH_2N, 2H); 4.2-4.5 (m, CH_2OCO + H [C9], 3 H); 5.2 (s [b], NH, 1 H); 7.2-7.9 (m, aromatic, 8 H);

Example 2:

Dichloro-N,N-diisopropylamino-phosphane

300 ml abs. ether, 81 ml anhydrous pyridine and 87.5 ml PCl_3 (1 Mol) are pre-cooled with stirring to -70°C in a 2 l three-neck, round-bottomed flask with a 500 ml dropping funnel, KPG stirrer, thermometer and acetone/dry ice bath. 142 ml diisopropylamine (1 Mol) in 250 ml abs. ether are added dropwise to it within 2 hours and the temperature is maintained at ca -60 to -65°C. After completing the addition, the thickened pulpy reaction mixture is allowed to reach room temperature and is diluted with about 600 ml abs. ether to make it more easily stirrable. After a further 3 hours stirring at room temperature the precipitate which forms is aspirated over a glass filter and washed several times with ether. After drawing off the ether at normal pressure, it is freed of unreacted PCl_3, diisopropylamine and pyridine in a water-jet vacuum and then the remaining oil is fractionally distilled in an

oil-pump vacuum (K_p 46°C/0.35 Torr). 73.4 g of the phosphane is obtained which corresponds to 36 % of the theoretical yield.

31P-NMR (ppm) ($CHCl_3$):167.5

Example 1:

2-(9-fluorenylmethoxycarbonyl-)aminoethyl-N,N-diisopropylamino-phosphochloridite

0.9 ml dichloro-N,N-diisopropylamino-phosphane (5 mmol) is dissolved in 30 ml abs. tetrahydrofuran in a 100 ml round-bottomed flask and 0.4 ml anhydrous pyridine is added to this. A solution of 1.4 g 2-(9-fluorenyl-methoxycarbonyl)aminoethanol (5 mmol) in 20 ml abs. tetrahydrofuran is added slowly dropwise to this mixture during ca 5 hours while stirring magnetically. After aspirating the pyridine hydrochloride which separates out and drawing off the tetrahydrofuran, the remaining oil, (2.2 g = 98 % of the theoretical yield) is used directly for the preparation of the nucleoside phosphoramidite (see Example 4).

Example 4:

5'-O-dimethoxytrityl-2'-deoxythymidine-3'-O-[2-(9-fluorenylmethoxycarbonyl)aminoethyl]-N,N-diisopropylamino-phosphane

a) 2.5 g 5'-O-dimethoxytrityl-2'-deoxythymidine (4.6 mMol) is dissolved in 50 ml dichloromethane (distilled over Na_2CO_3) as well as 2.5 ml N-ethyl-

N,N-diisopropylamine in a 100 ml round-bottomed flask. 2 ml 2-(9-fluorenylmethoxycarbonyl-)aminoethyl-N,N-diisopropylamino-phosphochloridite (ca. 5 mmol) is added to this using a disposable syringe. It is stirred for 48 hours at room temperature and evaporated down in a vacuum to a viscous residue.

The crude product is purified by chromatography on silica gel 60 (column 30 x 2 cm, mobile solvent petroleum ether 50 - 75°C/ethyl acetate/dichloromethane/pyridine = 4 : 8 : 8 : 2). The fractions containing product are collected and the solvent is completely removed in a vacuum.

0.9 g of a white foamy residue is obtained corresponding to 20 % of the theoretical yield.

b) In an alternative process 5.45 g 5'-O-dimethoxytrityl-2'-deoxythymidine (10 mmol) is dissolved with stirring in 100 ml absolute dioxan. A solution of 2.7 g bis-(diisopropylamino)-chlorophosphane (10 mmol) which was prepared according to S. Hammoto, H. Takaku, Chemistry Lett. 1986, 1401-1404 and 2.1 ml triethylamine (15 mmol) in 100 ml dioxan are added dropwise to this within 30 minutes. The reaction is followed by thin layer chromatography in methylene chloride/ethyl acetate = 1:1 as the mobile solvent. After 2 hours the precipitate of triethylammoniumchloride is filtered off under the protective gas argon and the filtrate is concentrated (colourless foam). The 5'-O-dimethoxytrityl-2'-deoxythymidine-3'-O-bis-(N,N-diisopropylamino)phosphane formed is converted to the desired product without further isolation. For

this the colourless foam is taken up in 100 ml absolute acetonitrile and 3 g 2-(9-fluorenyl-methoxycarbonyl)-amino-ethanol (Example 1) as well as 35 mg (5 mmol) tetrazole (sublimed) are added. It is stirred overnight at room temperature and the reaction is terminated by addition of 100 ml ethyl acetate. After extracting three times with saturated sodium chloride solution the combined organic phases are dried over sodium sulphate. After filtering off the sodium sulphate the filtrate is concentrated. The crude product is purified by chromatography on silica gel 60 H: (l = 24 cm, d = 4 cm; mobile solvent: methylene chloride/ethyl acetate = 5:1). After removal of the solvent a colourless foam is again obtained. This is taken up in 10 ml methylene chloride and precipitated with 400 ml ice-cold n-hexane. 1.8 g of the desired product is obtained as a colourless powder which corresponds to 20 % of the theoretical yield.

The two diastereomers can be distinguished by TLC as well as by 31P-NMR:

Rf-value (CH_2Cl_2/EA = 1:1) : 0.04, 0.15
31P-NMR (ppm) (CD_3CN) : 146.7, 145.8

Example 5

Synthesis of d ($Tp_{AE}TpTpTpTpTpTpTp_{AE}T$)

The synthesis of the oligonucleotide was carried out on a 1 µmol scale according to a standard protocol in a fully automated DNA synthesizer 8600 from the BioSearch

company. The synthesis instrument is equipped for this with a reaction column coated with 1 µmol thymidine carrier and in a first reaction step the 5'-OH protecting group (dimethoxytrityl-) is cleaved off by treatment with a 2 % dichloroacetic acid solution in dichloromethane. After washing the column with acetonitrile, the 5'-O-dimethoxy-triphenylmethyl-2'-deoxythymidine-3'-O-[2-(9-fluorenylmethoxy-carbonyl)aminoethyl]-N,N-diisopropylamino-phosphane of Example 4 modified at the P according to the present invention is coupled to the free 5'-OH group of the starting nucleoside and at the same time is activated with tetrazole in acetonitrile. The P atom which is still present in the trivalent form is converted after renewed washing into the natural pentavalent phosphate by oxidation with a solution of iodine in THF/lutidine/H_2O. The subsequent capping step with acetic anhydride/dimethylaminopyridine blocks the non-coupled 5'-OH-nucleoside by acetylation. By this means the formation of false sequences is suppressed. After washing, the synthesis cycle begins again from the start with a renewed cleavage of the 5'-O-dimethoxytrityl protecting group. In this way 6 thymidine building blocks with an unmodified phosphoramidite moiety are introduced into the reaction sequence, before in the last cycle a further coupling with the aminoethylated thymidine-phosphoramidite (Tp_{AE}) is carried out. After completion of the synthesis the oligonucleotide bound to the carrier is released by treatment with concentrated aqueous ammonia solution and at the same time the Fmoc protecting group of the aminoethylated phosphate is thereby removed. The result is 86 ODU/A_{260}. This crude mixture is processed by HPLC under the following conditions.

Column: Mono Q HR 10/10 (Pharmacia)
Eluant A (water), eluant B (0.5 n-LiCl)
Gradient: from A to 50 % B in 60 minutes.
The eluate is dialyzed overnight against H_2O
(Spektrapor,* MWCO 1000)

Yield: 55 ODU

Example 6:

Labelling of the oligonucleotide from Example 5 with digoxigenin

55 ODU/A_{260} of the oligomer from Example 5 is dissolved in 1 ml 0.1 m Na borate buffer pH 8.5 and mixed with a solution of 10 mg digoxigenin-O-succinyl-amidocaproic acid-N-hydroxysuccinimide ester in 1 ml dimethylformamide. The mixture is stirred for 18 hours at room temperature, evaporated to dryness in a vacuum, dissolved in H_2O and the mixture of products is separated by HPLC:

Column: Shandon Hypersil*ODS, 25 cm x 0.4 cm
Eluant A: 0.1 m triethylammonium acetate solution
Eluant B: 0.1 m triethylammonium acetate solution/ isopropanol
Gradient: from A to 50 % B in 30 minutes

The product fraction is concentrated by evaporation in a vacuum, taken up in water and dialyzed overnight against distilled water (Sprectrapor, MWCO 1000)

Yield: 11 ODU/A_{260}

* Trade Mark

Example 7:

Comparison of the detection limit in DNA tests

The hybridization properties of three identical oligonucleotides (38 mers) with a sequence specific for HIV was tested against a cloned HIV-DNA fragment (954bp PvuII/BglII fragment from the gag region of the HIV-Wfl.13-isolate). The oligonucleotides are labelled with digoxigenin at the following sites:

1. One each at a 5'-terminal uracil and at a uracil located in the middle (i.e. two dig labels, base label at C-5 of the uracil).

2. One each at a 5'-terminal, 3'-terminal and at a uracil located in the middle (three-fold dig label, base label at C-5 of the uracil).

3. One each at a 5'-terminal phosphate group and at a phosphate group located in the middle (2 dig labels/molecule, labelling according to the present invention).

a) Hybridization preparation for oligonucleotides with dig label

The sample DNA is either spotted directly onto filters in dilution series of 1 µl volume each or after separation in the agarose gel it is transferred by Southern blot using 20xSSC buffer onto the filters. The fixation is carried out by UV irradiation for 3 minutes.

The filters are pre-hybridized under the following conditions: 1 h at 40°C in 5xSSC, 0.5 % blocking reagent. The subsequent hybridization with dig labelled oligonucleotides is carried out under the following conditions: overnight at 4°C in 5xSSC, 0.5% blocking reagent, 200 ng oligonucleotide per ml hybridization solution.

The filters are washed afterwards for 4x10 min in 2xSSC, 0.1 % SDS at 40°C.

The detection is carried out analogous to the non-radioactive labelling and detection kit (Boehringer Mannheim GmbH) using a POD-labelled antibody against digoxigenin.

b) Results:

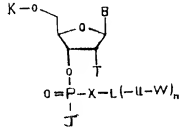
Detection limit of the spotted/blotted sample DNA

with a twice base labelled oligocleotide (1): 10 ng
with a three-fold base labelled oligonucleotide (2): 10 ng
with an oligonucleotide labelled twice via phosphate (3): 1-10 ng

The International (PCT) Application indicated herein is more fully identified below -
International Application WO 86/07363, published under the Patent Cooperation Treaty, filed June 13, 1986, D. L. Snitman e al, assigned to Amgen, published December 18, 1986.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A process for the production of a polynucleotide comprising a compound of the formula (V)



(V)

in which

K is hydrogen or the phosphorus atom of the phosphate residue a of a further nucleotide or polynucleotide,

J is a hydroxy group or a 5' oxygen atom of a further nucleotide or of a nucleotide sequence,

B is a natural or modified nucleic base,

T is hydrogen, lower alkyl, azide, lower alkyloxy or a hydroxyl group,

X is oxygen or sulphur,

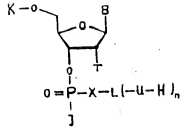
L is a (n+1) valent bridge number,

U is oxygen, sulphur, nitrogen or N-H,

W is a detectable residue or a residue which can be converted into a detectable residue and

n is a natural number from 1 to 200,

whereby more than one nucleotide component is modified on the phosphorus atom according to formula (V) by reaction of a polynucleotide comprising a compound of the formula IX,



(IX)

in which

K represents hydrogen or the phosphorus atom of the phosphate residue of a further nucleotide or polynucleotide,

J represents a hydroxy group or 5' oxygen atom of a further nucleotide or polynucleotide,

B represents a natural or modified nucleobase,

T represents hydrogen, lower alkyl, azide, lower alkyloxy or a hydroxy group,

X represents oxygen or sulphur,

L represents a (n+1) valcnt bridge number,

U represents oxygen, sulphur, nitrogen or N-H and

n represents a natural number from 1 to 200,

whereby more than one nucleotide component is modified on the phosphorus atom according to formula IX with a compound of the formula IV,

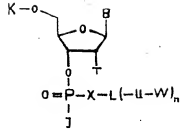
Y-W (IV),

in which

Y is a reactive group and

W is a detectable residue or a residue which can be converted into a detectable residue.

2. A polynucleotide comprising a compound of the formula (V)



(V)

in which

K is hydrogen or the phosphorus atom of the phosphate residue of a further nucleotide or of a polynucleotide,

J is a hydroxy group or a 5' oxygen atom of a further nucleotide or of a polynucleotide,

B is a natural or modified nucleic base,

T is hydrogen, lower alkyl, azide, lower alkyloxy or a hydroxyl group,

X is oxygen or sulphur,

L is a (n+1) valent bridge number,

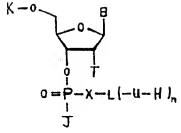
U is oxygen, sulphur, nitrogen or N-H,

W is a detectable residue or a residue which can be converted into a detectable residue and

n is a natural number from 1 to 200,

whereby more than one nucleotide component is modified on the phosphorus atom according to formula V.

3. A process for the production of polynucleotides comprising compounds of the formula (IX)



(IX)

in which

K represents hydrogen or the phosphorus atom of the phosphate residue of a further nucleotide or of a polynucleotide,

J represents a hydroxy group or a 5' oxygen atom of a further nucleotide or of a polynucleotide,

B represents a natural or modified nucleobase,

T represents hydrogen, lower alkyl, azide, lower alkyloxy or a hydroxy group,

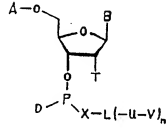
X represents oxygen or sulphur,

L represents a (n+1) valent bridge number,

U represents oxygen, sulphur, nitrogen or N-H and

n represents a natural number from 1 to 200,

by reaction of a compound of the formula I



(I)

in which

A represents an oxygen protecting group, a nucleotide or an oligonucleotide,

B represents a natural or modified nucleic base,

X represents oxygen or sulphur,

L represents a (n+1) valent bridge number,

T represents hydrogen or a hydroxy group which is protected if desired,

U represents oxygen, sulphur, nitrogen or N-H,

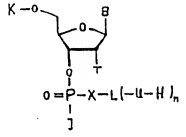
V represents a protecting group which can be cleaved off,

n represents a natural number from 1 to 200 and

D represents a secondary amine residue,

with a further nucleoside which has a free 5'-hydroxyl group and oxidation of the polynucleotide formed.

4. A polynucleotide comprising compounds of the formula (IX)



(IX)

in which

K represents hydrogen or the phosphorus atom of the phosphate residue of a further nucleotide or of a polynucleotide,

J represents a hydroxy group or a 5' oxygen atom of a further nucleotide or of a polynucleotide,

B represents a natural or modified nucleobase,

T represents hydrogen, lower alkyl, azide, lower alkyloxy or a hydroxy group,

X represents oxygen or sulphur,

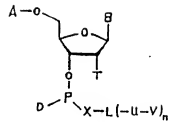
L represents a (n+1) valent bridge number,

U represents oxygen, sulphur, nitrogen or N-H and

n represents a natural number from 1 to 200,

whereby more than one nucleotide component is modified on the phosphorus atom according to formula IX.

5. A nucleoside phosphoramidite of the formula (I)



(I)

in which

A represents an oxygen protecting group, a nucleotide or an oligonucleotide,

B represents a natural or modified nucleobase,

X represents oxygen or sulphur,

L represents a (n+1) valent bridge number,

T represents hydrogen, lower alkyl, azide, lower alkyloxy or a possibly protected hydroxy group,

U represents oxygen, sulphur, nitrogen or N-H,

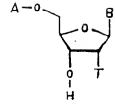
V represents a protecting group which can be cleaved off,

n represents a natural number from 1 to 200 and

D represents a secondary amine residue.

6. Use of a nucleoside phosphoramidite of the formula (I), as defined in claim 5, for the production of a corresponding compound of formula (V) as defined in claim 2.

7. A process for the production of a nucleoside phosphoramidite of the formula (I), as defined in claim 5, by reaction of a compound of the formula (II)



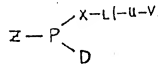
(II)

in which

A represents an oxygen protecting group, a nucleotide or an oligonucleotide,

B represents a natural or modified nucleic base and

T represents hydrogen, lower alkyl, azide, lower alkyloxy or a possibly protected hydroxy group, with a phosphane of the formula III



(III)

in which

Z represents a readily removable group,

X represents oxygen or sulphur,

L represents an at least bivalent bridge number,

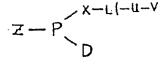
U represents oxygen, sulphur, nitrogen or N-H,

V represents a protecting group which can be cleaved off,

n represents a natural number from 1 to 200 and

D represents a secondary amine residue.

8. A phosphane of the formula (III)



(III)

in which

Z represents an halogen,

X represents oxygen or sulphur,

L represents an at least bivalent bridge number,

U represents oxygen, sulphur, nitrogen or N-H,

V represents a protective group which can be cleaved off,

n represents a natural number from 1 to 200 and

D represents a secondary amine residue.

9. A process for the production of phosphanes, wherein a compound of the formula (VI)

P(-Z)3 VI)

in which Z represents a readily removable group, said formula (VI) compound is reacted with a secondary amine of the formula (VII)

H-D VII)

in which

D represents a secondary amine residue

and the product is allowed to react with a compound of the formula VIII

H-X-L(-U-V)n (VIII)

in which

X represents oxygen or sulphur,

L represents a (n+1) valent bridge number,

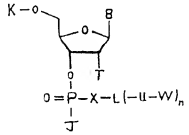
U represents oxygen, sulphur, nitrogen or N-H,

V represents a protecting group which can be cleaved off,

n represents a natural number from 1 to 200 and

the resulting product is isolated.

10. Use of a polynucleotide comprising a compound of the formula (V)



(V)

in which

K represents hydrogen or the phosphorus atom of the phosphate residue of a further nucleotide or polynucleotide,

J represents a hydroxy group or a 5' o oxygen atom of a further nucleotide or of a polynucleotide,

B represents a natural or modified nucleic base,

T represents hydrogen, lower alkyl, azide, lower alkyloxy or a hydroxyl group,

X is oxygen or sulphur,

L is a (n+1) valent bridge number,

U is oxygen, sulphur, nitrogen or N-H,

W is a detectable residue or a residue which can be converted into a detectable residue and

n is a natural number from 1 to 200,

whereby more than one nucleotide component according to formula V is modified on the phosphorus atom, for the detection of a nucleotide sequence which is substantially complementary to this polynucleotidc.

11. A reagent for the detection of a nucleic acid in a sample by bringing the sample into contact with a nucleic acid which is substantially complementary thereto, wherein said reagent comprising said nucleic acid complementary to the sample nucleic acid and wherein said nucleic acid

complementary to the sample nucleic acid containing a polynucleotide according to claim 2.

12. Use of a polynucleotide comprising a compound of the formula (V), as defined in claim 2, as primer in the enzymatic synthesis of double-stranded nucleic acids.

13. A process of claim 1, wherein L is a linear saturated hydrocarbon residue containing 1 to 10 carbon atoms.

14. A process of claim 1 or 13, wherein n is 1 to 3.

15. A process of claim 1 or 13, wherein n is 1.

16. A process of claim 1 or 13, wherein W is a hapten residue.

17. A process of claim 3, wherein said further nucleoside is bound to a solid phase.

18. A process of claim 17, wherein V is a protecting group which is selectively cleaved off under conditions under which the compound of formula (IX) is cleaved from said solid phase, and simultaneously therewith.

19. A process of claim 18, wherein V is a protecting group which is cleaved off under alkaline or ammoniacal conditions.

20. A process of claim 3, wherein T is hydrogen.

21. A process of claim 3, wherein X is oxygen.

22. A process of claim 3, wherein U is N -H.

23. A process of claim 17 wherein A is an oxygen protecting group.

24. A process according to claim 23, wherein after said reaction of the compound of formula (I), the oxygen protecting group is split off and the resulting compound is reacted with a further mononucleoside phosphoramidite or a compound of said formula (I).